NOAA-NATIONAL OCEAN SERVICE

CENTER FOR COASTAL ENVIRONMENTAL HEALTH AND BIOMOLECULAR RESEARCH

FY00 SIGNIFICANT ACCOMPLISHMENTS

MARINE BIOTOXINS PROGRAM

CHARACTERIZATION OF BACTERIA ALGICIDAL TO HARMFUL ALGAL SPECIES

We are continuing to examine interactions between algicidal bacteria and harmful algal species as a means to understand the microbial processes influencing bloom population dynamics. Denaturing gradient gel electrophoresis and fluorescent in-situ hybridization with oligonucleotide probes are being used to assess changes in microbial communities associated with *G. breve* following the introduction of algicidal bacteria to algal cultures. Following inoculation into bacteria-containing *G. breve* cultures: 1.) algicidal bacteria numbers rapidly increase; 2.) the microbial assemblage changes, with some bacterial taxa disappearing and others appearing throughout the killing event; and, 3.) algicidal activity of introduced bacteria seems to vary according to the target alga's physiological status, with resistance to attack decreasing with declining algal growth rate. Our data suggest that algicidal bacteria may be able to fill a vacant niche (or displace another organism from its niche) within a *G. breve* culture. Use of these molecular approaches will aid in determining whether similar processes occur during a natural *G. breve* bloom.

ALGAL GROWTH REGULATION AND SIGNALLING

The objectives of ongoing algal growth regulation and cell signaling studies on dinoflagellates are to better understand cellular mechanisms regulating dinoflagellate blooms formation and to identify potential molecular targets for control measures. Studies carried out this year focussed on the Florida red tide dinoflagellate, *G. breve*. In a continuation of cell cycle regulation studies, this year we have identified the presence of cyclin in *G. breve* using western blotting techniques and immunocytochemistry. This, in conjunction with our previous work on cyclin dependent kinase, defines the fundamental cell cycle regulatory apparatus in *G. breve*. A cDNA library has been made and is being screened for the cyclin gene(s). We have completed studies that identify a eukaryotic type cyclic AMP signaling pathway in dinoflagellates. A cyclic AMP dependent protein kinase in *A. operculatum* has been identified and its subunit structure characterized biochemically. Studies were initiated this year on stress proteins in *G. breve*. We have identified the presence of the chloroplast small heat shock protein (hsp) and hsp60. These studies will be continued in the next FY to identify their involvement in bloom termination and light, salinity, and temperature stress.

POTENTIAL TOXICITY OF *PFIESTERIA*-LIKE DINOFLAGELLATES ISOLATED FROM FLORIDA

Cultures of different species of "Cryptoperidiniopsoid" dinoflagellates were grown under controlled conditions. Each culture was identified via scanning electron microscopy at the Florida Marine Research Institute before shipment to the Marine Biotoxin Program. Each strain was re-identified after mass culture and toxin analysis. Strains were grown in 100 L batches cultures and harvested at late-log growth phase. Production of biological active substances by each culture was examined from both resulting cell mass and spent culture medium. Both cell mass and spent culture medium were passed though a silica column and eluted with an elutropic solvent series. Totals of 5 samples were collected for both the cell mass extract and spent culture medium. Each of the 10 extracts was tested for the possibility of bioactivity using both live assays and cell based assays. Live bioassays included brine shrimp and sheepshead minnows while cell based assay included the GH4C1 cytotoxicity assay. Solvent fractionation yielded several fractions that were active. A non-polar fraction was active on the shrimp bioassay and the sheepshead minnow bioassay. Subsequent structural analysis of this fraction showed this activity in part was due to DEHP, a man-made phthalate ester. This and other fractions are still under pharmacological characterization. A polar fraction was active on the brine shrimp bioassay and the cytotoxicity assay but was inactive on the sheepshead minnow assay. This data provides initial evidence of bioactive substances from cultures of Cryptoperidiniopsoid.

Whether this organism produces a toxic substance is presently unknown and will require future pharmacological and chemical investigations.

PREPARATIVE ISOLATION OF *PFIESTERIA* TOXINS FROM CULTURE

Methodology for the reproducible isolation of Pfiesteria toxins has been developed and employed for preparative toxin production. This methodology allows toxic fractions to be isolated in a stable form providing a relatively neutral environment free of matrix buffers, enzymes and so forth. Such methodology now enables us to rapidly isolate the toxins from mass culture, quickly removing the toxin(s) from oxidative or reductive environments that would otherwise degrade or destroy the toxin. Purified extracts obtained from this method are being used for toxin characterization both in terms of biological activity as well as molecular characterization using Nuclear Magnetic Resonance and Mass Spectrometry. A lipohpillic toxic fraction has been identified by NMR and GC-MS as bis(diethylhexyl) phthalate, a common plasticizer. The major source for this material has been identified as Instant Ocean. No other non-polar toxin has been observed. The characterization of the polar toxin(s) is well underway.

RECENT ADVANCES IN AND APPLICATIONS FOR A PSP RECEPTOR BINDING ASSAY

Several years ago we described a high throughput receptor binding assay for PSP toxins and its use for detecting toxic activity in shellfish and algal extracts. We have since increased the assay efficiency through application of microplate scintillation technology (4h turn around time), and have validated use of 11-[^{3}H]-tetrodotoxin as an alternative radioligand to the [^{3}H]-saxitoxin conventionally employed in the assay. Efforts are now focused on identifying the applications for which the receptor assay can provide data comparable to the more time consuming, technically demanding HPLC analysis of PSP toxins. We have compared the results of both methods for toxic dinoflagellates, field samples of *Alexandrium* spp. and its associated zooplankton grazers, as well as contaminated human fluids from a PSP outbreak. In general, receptor-based STX equiv. values were highly correlated and in close quantitative agreement with those produced by HPLC. While the receptor binding assay does not provide toxin composition data obtainable by HPLC, it does represent a robust and reliable means of rapidly assessing PSP-like toxicity in laboratory and field samples. Moreover, this assay should be effective as a screening tool in suspected cases of PSP intoxication.

ASSAY VALIDATION AND TECHNOLOGY TRANSFER

As part of the U.N. sponsored technology transfer program on red tides in SE Asia, we conducted a training workshop on receptor assays in Manila, Philippines in December 1999. The workshop was attended by 14 particiants from 7 SE Asian countries. In addition, this year we hosted two individuals associated with this program for extensive receptor assay training in the laboratory: Ms. Cecilia Conaco, of the University of the Philippines (October-Nov 1999) and Ms. Mei Mei Ch'ng, of University of Malaysia (March – August 2000). We will host up to 3 additional personnel from participating nations during FY 2001. The program will then carry out a round robin interlaboratory comparison trial between participating nations in 2002. A receptor assay training workshop was held in May at CCEHBR to transfer this technology to representatives of two state regulatory agencies interested in its potential as a replacement for the mouse bioassay: California Dept. of Health and Florida DNR.

TANDEM MASS SPECTROMETRIC IDENTIFICATION OF DOMOIC ACID: IDENTIFICATION/QUANTITATION OF DOMOIC ACID IN SCALLOPS FROM THE WEST COAST OF SCOTLAND

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used in the analysis of domoic acid (DA) in scallops collected during the late 1999 / early 2000 from the West Coast of Scotland. The DA analysis of the scallops was in support of efforts by collaborators at the Scottish Association for Marine Science - Dunstaffnage Marine Laboratory to characterize the dramatic increase in amnesic shellfish poisoning (ASP) on the Scottish west coast. The LC-MS/MS method allowed detection of DA in a tissues excised from the scallops. This utilization of LC-MS for domoic acid identification provided the absolute identification of DA in the sampled scallops that would not be accessible from functional assays or other HPLC-coupled detection methods. Results of the LC-MS/MS analyses indicated some of the highest ever recorded levels of DA from shellfish and provide valuable data in support of oceanographic modelling studies concerning the influence of the rapidly expanding Scottish aquaculture industry on the dynamics of harmful algal bloom on the west coast of Scotland.

ACCUMULATION OF PSP TOXINS IN ZOOPLANKTON GRAZERS

Algal toxins are well-known to undergo trophic transfer and accumulation in marine food webs, causing intoxication of upper-level consumers such as fish, sea birds, and marine mammals. Work in collaboration with investigators at U. Mass Dartmouth and the Woods Hole Oceanographic Institution has focused on the tracking movement of PSP toxins from their algal producers into the associated zooplankton grazer assemblage. Through receptor assay-based analysis of algal and zooplankton size fractions from Massachusetts Bay, MA, we determined that PSP toxins did, indeed, accumulate in the grazers and that toxicity was disproportionately concentrated in the larger zooplankton size fractions (200-500 : m, > 500 : m). Interestingly, these size fractions, frequently dominated by large copepods, comprised only a small portion of total zooplankton abundance. These larger toxin-accumulating copepods could thus provide a direct trophic linkage for PSP intoxication of marine mammals such as baleen whales, which are known to preferentially feed upon these grazers.

DETERMINATION OF DOMOIC ACID IN MOLE CRABS (*Emerita analoga*): A POSSIBLE NEW INDICATOR SPECIES

We are currently examining the trophic transfer of domoic acid (DA) through the pelagic and benthic food webs in Monterey Bay, California, a site of recurrent *Pseudo-nitzschia* blooms and marine mammal mortalities associated with these HAB events. One aspect of this work, being done in collaboration with CA State Univ. at Monterey Bay and the Univ. of CA at Santa Cruz, is to evaluate use of the mole crab (*Emerita analoga)*, as an indicator species for DA, since the current sentinel organism, the intertidal blue mussel (*Mytilus californianus*), shows minimal or undetectable toxicity during local bloom events. We have developed a new extraction protocol for the determination of DA in mole crabs that yields toxin recoveries of 96.5 $\pm$ 2.9 percent. We have also confirmed by LC-MS/MS that mole crabs accumulated measurable amounts of DA during toxic *Pseudo-nitzschia* bloom events (0.5-10 : g DA/g tissue), while the blue mussel showed no detectable toxin. In addition, the rise and fall of DA toxicity in mole crabs coincided with similar changes in *Pseudo-nitzschia* cell concentrations. Extensive field trials are now underway to further compare the DA toxicity of mole crabs vs. blue mussels and its correlation to the presence of toxic *Pseudo-nitzschia* cells, and to critically evaluate the potential use of mole crabs as the sentinel species for coastal DA events in this area. These data will also be useful in establishing the effectiveness of mole crabs as a vector for transferring DA to higher trophic levels.

BIOMONITORING HAWAIIAN GREEN SEA TURTLES (*Chelonia mydas*) FOR THE PRESENCE OF THE TUMOR PROMOTER OKADAIC ACID

Fibropapillomas are begin tumors, which in sea turtles are increasing in occurrence. These tumors are fibrous masses of tissue often growing on the eyes, mouth or flippers and on occasions internally in lungs and kidney. These tumors can impede seeing and feeding, are at times fatal and are a threat to recovering species. A joint project with NMFS-Hawaii laboratory was initiated to biomonitor green sea turtles in regions where fibropapillomas are prevalent using the newly developed blood collection card sampling method. The monitoring is conducted for okadaic acid, a dinoflagellate toxin that is a known first stage tumor promoter. To optimize detection limits, a fluorescent microplate assay has been optimized for use with the rapid blood extraction from the collection cards. Regular sampling and analysis is continuing and positive samples will be related to the disease state of the animals. Blood collection cards have permitted the biomonitoring of toxin in living animals and has greatly increased sampling ease in the field.

IDENTIFICATION OF BREVETOXIN IN BOTTLENOSE DOLPHINS FOLLOWING THE GULF OF MEXICO MORTALITY EVENT

Using brevetoxin (PbTx) receptor assays as a rapid screen and HPLC-MS/MS for chemical confirmation, we were able to demonstrate the presence of PbTx in livers of dolphins that died in the course of a *G. breve* bloom in the Florida panhandle region. From August 1999 until February 2000, over 120 bottlenose dolphins stranded in the Florida panhandle. This number represents more than a 4 fold increase from historic annual averages of bottlenose dolphin stranding for this area. In August - October, the strandings were principally localized in the Appalachicola area, concurrent with a bloom of *G. breve*. No strandings occurred in November. However, strandings again increased during December through February 2000 and were localized in the western counties, primarily in the Choctawhatchee Bay area, where a *G. breve* bloom persisted. Brevetoxin was previously implicated in the 1996 manatee epizootic. Levels of PbTx found in dolphin livers were similar to those reported in manatees in 1996.

IDENTIFICATION OF DOMOIC ACID IN GREY WHALES AND SEA LIONS IN CALIFORNIA COASTAL WATERS

Using DA receptor assays as a rapid screen and HPLC-MS/MS for chemical confirmation, we were able to confirm that stranded gray whales and sea lions were exposed to DA at concentrations that might be expected to cause adverse effects. We previously demonstrated that domoic acid was the causative agent of a California sea lion mortality event in 1998. This year for the first time, DA poisoning appears to have had widespread impacts on several different marine mammal species with diverse feeding habits and geographic distributions: gray whales stranded in San Francisco Bay in April-May, sea otters that died in the same area during the same timeframe, sea lions stranded in San Luis Obispo County in June-early July, and sea lions stranded 100 miles farther south in Ventura/Los Angeles Counties in late July. Most of the sea lions were in good body condition, but displayed seisure and scratching activities documented previously in the 1998 sea lion mortality event. Blooms of the DA producing diatom *Pseudonitzschia australis* were found in California coastal waters concurrently with the marine mammal morbidities/mortalities.

RECEPTOR IDENTIFIED FOR PUTATIVE *PFIESTERIA* TOXIN PROVIDES INSIGHT INTO EFFECTS OF PFIESTERIA ON HUMANS AND FISH

The pharmacologic activity of a putative toxin (pPfTx) produced by *P. piscicida* has been examined by characterization of the signaling pathways that induce the c-fos luciferase construct in GH_4C_1 rat pituitary cells. A class of purinergic receptors mediates this c-fos pathway with analog selectivity and functional ionic conductances consistent with a purinergic receptor of the P2X7 class. The irreversible P2X7 antagonist, adenosine 5'-triphosphate-2',3'-dialdehyde, was used to demonstrate that the pPfTx requires this pathway for activation. P2X7 receptors are found predominantly on myeloic cells including mature macrophages, mast cells and microglial cells. A role of P2X7 receptors in the action of pPfTx is of interest**,** in consideration of the fact that this toxic dinoflagellate has been reported to cause a range of health impacts in both finfish and humans. The effects linked to *Pfiesteria* toxicity may be related to an inflammatory response, either in macrophages in the periphery or microglia in brain tissue. Implication of P2X7 receptors as a potential target for the bioactive substance produced by toxic *P. piscicida* provides a common basis for the investigation of symptoms that previously have been regarded as unrelated, such as ulcers in menhaden and cognitive dysfunction in humans.